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Genistein Reduces Tumor Necrosis Factor α–Induced Plasminogen Activator Inhibitor-1 Transcription but not Urokinase Expression in Human Endothelial Cells

By Victor W.M. van Hinsbergh, Mario Vermeer, Pieter Koolwijk, Jos Grimbergen, and Teake Kooistra

The plasminogen activator inhibitor PAI-1 is markedly elevated in vivo and in vitro upon exposure to the inflammatory mediators tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and bacterial lipopolysaccharide. Here we report that the isoflavone compound genistein prevents the increase in synthesis of PAI-1 induced by these inflammatory mediators in human endothelial cells in vitro, and partially reduces the basal PAI-1 production by these cells. These effects of genistein were accompanied by a decrease in PAI-1 mRNA and in a suppression of the PAI-1 transcription rate as shown by run-on assay. A specific action of genistein, probably by inhibiting a tyrosine protein kinase, is likely, because the structural genistein analogue daidzein, which has a low tyrosine protein kinase inhibitor activity, did not inhibit PAI-1 synthesis. Vanadate, a tyrosine protein phosphatase inhibitor, increased PAI-1 production. The effect of genistein on PAI-1 synthesis was rather selective. Herbimycin A also reduced PAI-1 synthesis, but several other tyrosine protein kinase inhibitors, namely tyrphostin A47, methyl-2,5-dihydroxy-cinnamate, and compound 5, were unable to do so. All these tyrosine protein kinase inhibitors reduced basic fibroblast growth factor (b-FGF)–induced [^{3}H]thymidine incorporation in endothelial cells. This indicates that the effect of genistein on PAI-1 transcription proceeds independently of its effect on mitogenesis. In contrast to TNFα-induced PAI-1 production, the transcription and synthesis of urokinase-type plasminogen activator (u-PA) was not inhibited by genistein. A TNFα-mutant (Trp^{32}Thr^{86}TNFα) that specifically recognizes the 55-kD TNF-receptor, mimicked the effects of TNFα on both PAI-1 and u-PA. Because genistein affected PAI-1, but not u-PA induced by this mutant, involvement of different TNF-receptors cannot underlie the difference in the effects of genistein on PAI-1 and u-PA synthesis. Because genistein also inhibited PAI-1 induction by thrombin and IL-4, it is likely that genistein does not act on a TNFα-receptor–coupled protein kinase but on the signal transduction pathway enhancing PAI-1 transcription. Our results suggest that the TNFα-induced signal transduction pathway of PAI-1 transcription involves a genistein-sensitive step that is not involved in the induction of u-PA by TNFα. Given the limited sensitivity to several other tyrosine protein kinase inhibitors, this genistein-sensitive step may be a potential target for pharmacologic intervention to reduce elevated plasma PAI-1 levels.

PLASMINOGEN ACTIVATORS regulate fibrinolysis[1,2] and play an important role in local proteolytic processes, which occur in cell migration, cell invasion, and angiogenesis.[3-5] Plasminogen activation is controlled by cellular receptors and specific inhibitors, in particular plasminogen activator inhibitor-1 (PAI-1).[6,7] PAI-1 is synthesized in the vascular wall and, depending on the species, in the liver. It is also present in blood platelets from which it can be released upon activation. Plasma concentrations of PAI-1 are elevated in sepsis,[8] postoperatively,[9] and in patients with an increased risk of recurrent myocardial infarction.[10] PAI-1 protein and mRNA are also enhanced in arteriosclerotic blood vessels.[11,12] A number of components including bacterial lipopolysaccharide (LPS), the inflammatory mediators tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), thrombin, transforming growth factor β (TGFβ), and oxidized lipoproteins enhance the synthesis of PAI-1 by endothelial cells in vitro.[13-19] LPS, IL-1, and TNFα also increase the plasma concentration of PAI-1 in vivo.[13,14,16,19-21] This is predominantly associated with an increased synthesis of PAI-1 in the endothelial cells.[22,23]

Because an elevated concentration of PAI-1 in plasma can decrease the fibrinolytic activity in blood or in thrombi and has been associated with the increased risk of thrombus formation in sepsis, it could be of clinical importance to reduce PAI-1 production. However, no drugs are available yet, and insight into the regulation of PAI-1 synthesis is still insufficient to design such a drug. In a study on the effects of basic fibroblast growth factor (b-FGF) and TNFα on the synthesis of u-PA and PAI-1, we observed that the isoflavone compound genistein inhibited TNFα-induced PAI-1 production (see below). Two recent studies also showed that endothelial PAI-1 production, which was enhanced by insulin[24] or by b-FGF,[25] was inhibited by genistein in vitro. No effect of genistein on PAI-1 mRNA was observed,[24] and the effect of genistein was largely attributed to inhibition of the tyrosine kinase activity of the receptor for insulin or b-FGF, respectively. In the present report we describe the inhibitory effect of genistein on the TNFα-enhanced PAI-1 transcription and synthesis in human endothelial cells. Our data point to a specific TNFα-induced signal transduction pathway enhancing PAI-1 transcription, which is inhibited by genistein but not by several other tyrosine protein kinase inhibitors, and which is not involved in TNFα-induced urokinase-type plasminogen activator (u-PA) production.

MATERIALS AND METHODS

Materials. Human recombinant TNFα[26] was a gift from Jan Tavernier (Biogent, Gent, Belgium); the TNFα preparation contained 2.45×10^7 U/mL mg protein and less than 40 ng LPS per milligram of protein. A human recombinant TNFα-mutant Trp^{32}Thr^{86}TNFα (Ro-45-2089/000), which interacts only with the 55-kD TNF-recep-

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tor,[27] was a gift from Dr Werner Lesslauer (Hoffmann-LaRoche, Basel, Switzerland); the preparation contained 7.6 endotoxin units (EU) LPS per milligram of protein of TNFα mutant. IL-4[28] was a gift from René de Waal Malefyt (DNAX, San Francisco, CA); the IL-4 preparation had a specific activity of 10^7 U/mg protein. LPS of *Escherichia coli* and bovine α-thrombin were purchased from Sigma (St Louis, MO). Genistein, daidzein, tyrphostin A47, herbimycin A, compound 5, methyl-2,5-dihydroxy-cinnamate, and sodium orthovanadate were obtained from LC Laboratories (Woburn, MA). The pUK 0321 harboring a 1,023-bp fragment of the human u-PA cDNA[29] was a gift from Dr Wolf-Dieter Schleuning (Schering AG, Berlin, Germany); the PAI-1 cDNA probe,[30] the t-PA cDNA probe,[31] the glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe,[32] and the actin cDNA probe[33] were described previously. The u-PA receptor cDNA probe[34] was a gift from Drs Francesco Blasi and Kjeld Danø (Copenhagen, Denmark); the 55-kD TNF receptor cDNA probe (Gen EMBL M33480) and the 75-kD TNF receptor cDNA probe (Gen EMBL X54716) were kindly provided by Dr Werner Lesslauer. Human recombinant b-FGF, Proteinase K, and Sephadex G50 (fine) columns were obtained from Boehringer Mannheim (Mannheim, Germany); DNAase I was from Bethesda Research Laboratories (Bethesda, MD). Hybond-N$^+$ filters, Hyperfilm MP, and [α^{32}P]UTP, [α^{32}P]dCTP, and [^{3}H]thymidine were obtained from Amersham (Amersham, Bucks, UK).

Isolation and culture of endothelial cells. Endothelial cells from human umbilical vein were isolated, cultured, and characterized as previously described.[35] Cells were incubated in M199 medium supplemented with 20 mmol/L HEPES (pH 7.3), 10% pooled human serum, 10% heat-inactivated newborn calf serum, 150 μg/mL crude endothelial cell growth factor,[36] 5 U/mL heparin, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2/95% air atmosphere. Conditioned media were obtained after 24 hours incubation of confluent cells (after 1 or 2 passages) in M199 medium, supplemented with 20 mmol/L HEPES (pH 7.3), 10% pooled human serum, penicillin/streptomycin, and the indicated agents. They were immediately centrifuged and stored at −20°C until assay of fibrinolysis proteins.

Incorporation of [^{3}H]thymidine. Incorporation of [^{3}H]thymidine in DNA was determined as previously described.[37] Confluent cultures of endothelial cells were detached by trypsin/EDTA solution, and seeded at a density of 10^4 cells per cm^2 on fibronectin-coated dishes cells in M199-HEPES medium supplemented with 10% heat-inactivated newborn calf serum and penicillin/streptomycin with or without 10 ng/mL b-FGF. After a preincubation period of 18 hours, a tracer amount of [^{3}H]thymidine (0.5 μCi per 2 cm^2 well, added in a 10-μL volume) was added and the cells were incubated for another 6-hour period. Subsequently, the cells were washed with phosphate-buffered saline; and [^{3}H]-labeled DNA was precipitated in 10% trichloroacetic-acid, washed, washed twice in 96% ethanol, dissolved in 0.3 mL 0.3 mol/L NaOH, and counted in a liquid scintillation counter.

RNA isolation and Northern blotting. Total RNA was isolated according to the method of Chomczynski and Sacchi.[38] Formaldehyde-agarose gel electrophoresis, Northern blotting, and hybridization were performed as previously indicated.[30]

Isolation of nuclei and run-on experiments. Rate of transcription was estimated from run-on experiments, which were conducted essentially as described by Groudine et al,[39] with minor modifications.[40] To that end, the cells of 12 confluent primary cultures were detached and pooled, and subcultured; tight confluent monolayers of cells (after two passages) were used. For each nuclear preparation 324 cm^2 of confluent endothelial cells were used. These cells were washed and incubated for 4 hours in culture medium supplemented with 500 U/mL TNFα or without this cytokine. Subsequently the cells were washed, scraped, and centrifuged, and the nuclei were isolated as previously described and stored at −80°C until use.[40]

Details of the subsequent RNA labeling during run of transcription of the isolated nuclei, and the isolation of the labeled RNA are given by Twisk et al.[40] Incorporation of the label was measured by liquid-scintillation counting, and equal amounts of labeled RNA were added to the filters for hybridization.

Target DNA, which was 10 μg of linearized plasmid material containing cDNA sequences of human PAI-1, u-PA, tissue-type plasminogen activator (t-PA), u-PA receptor, 75-kD TNF receptor, 55-kD TNF receptor, actin, or GAPDH, were slot-blotted onto strips of Hybond-N$^+$ filter and cross-linked with 0.4 mol/L NaOH for 30 minutes. The filters were preincubated for 30 minutes at 65°C in a 0.5 mol/L sodium phosphate buffer (pH 7.5) containing 7% (wt/vol) sodium dodecyl sulfate (SDS) and 1 mmol/L EDTA. Next, they were hybridized with the labeled RNA for 36 hours in the same buffer. The various filters were washed once for 5 minutes and twice for 30 minutes in 2× SSC/1% SDS at 65°C, and exposed to Hyperfilm MP together with an intensifying screen (Eastman Kodak Co, Rochester, NY) for 2 to 14 days at −80°C.

Quantification of RNA. The relative amounts of RNA on the Northern blots and hybridized after run-on assay were quantified from adequately exposed autoradiographs by scanning with a Shimadzu CS 910 chromatograph scanner (Shimadzu, Kyoto, Japan) and integration of the areas under the curves by using a data processor, as described by Twisk et al.[40]

Assays. Levels of PAI-1 antigen in endothelial cell conditioned media were assayed by enzyme-linked immunosorbent assay (ELISA) (IMULYSE PAI-1) obtained from Biopool (Umeå, Sweden), according to the manufacturer's description.

Assay of t-PA antigen was performed with the ELISA Thrombonostika t-PA (Organon-Teknika, Turnhout, Belgium) as described by Bos et al.[41] In this assay, free t-PA and t-PA:PAI-1 complexes are detected with equal efficiency.

The amount of u-PA antigen was determined by ELISA using two monoclonal antibodies (MoAbs) (UK 2.1 and UK 26.15 recognizing different epitopes on the u-PA antigen) as catching antibodies and a biotinylated MoAb (LMW 11.1) as a tagging antibody. The monoclonals were raised and characterized in our laboratory. Ukidan two-chain u-PA (Serono, Aubonne, Switzerland) was used as a standard. The assay determines sc-u-PA, active tc-u-PA, and u-PA:PAI-1 complex with equal efficiency.

Cells were counted in a hemocytometer after detachment by trypsin/EDTA treatment.

Statistics. Statistical significance was analyzed by the Student's *t*-test for paired data and accepted at $P < .05$.

RESULTS

The inflammatory mediators TNFα, IL-1, and LPS induce a several-fold increase in PAI-1 synthesis in human endothelial cells. When the cells were simultaneously incubated with genistein, this increase was prevented in a concentration-dependent way (Fig 1, A and B; Table 1). Half-maximal inhibition was observed at about 20 μg/mL genistein (75 μmol/L). Daidzein (10 to 100 μg/mL), a structural analogue of genistein with a much lower tyrosine kinase inhibitory activity,[42] did not reduce the induction of PAI-1 by inflammatory mediators (not shown). Genistein had a smaller but consistent suppressive effect on the basal production of PAI-1 antigen (Fig 1A, Table 1). Increases in PAI-1 synthesis induced by IL-4 (Table 1) or thrombin (1 U/mL, not shown) were inhibited to a similar extent as those induced by the inflammatory mediators TNFα, IL-1α, and LPS, but gen-

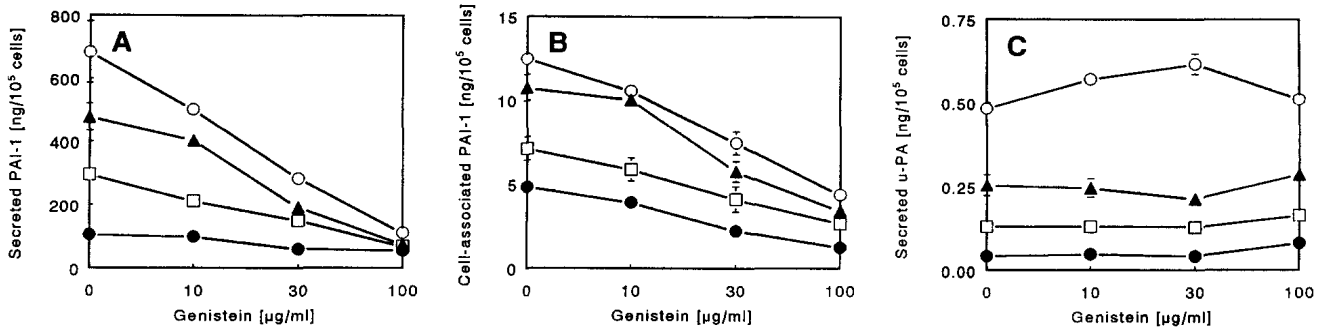

Fig 1. Genistein inhibits PAI-1 synthesis in a concentration-dependent way. PAI-1 and u-PA antigens were assayed in conditioned medium (A and C) and 0.5% Triton X-100 cell extracts obtained after 24-hour incubation of human umbilical vein endothelial cells (confluent, after one passage) in M199-10% human serum supplemented with 500 U/mL TNFα (○), 5 U/mL IL-1α (□), 10 μg/mL LPS (▲), or without addition of inflammatory mediator (●). Genistein was added 1 hour before the incubation, and remained present throughout the 24-hour period. PAI-1 and u-PA antigens were determined as indicated in Materials and Methods. u-PA antigen values in the cell extracts were all less than 0.1 ng/10⁵ cells.

istein did not reduce the basal or stimulated production of u-PA (Fig 1C, Table 1). Genistein had no effect on the basal t-PA production. However, the TNFα- and LPS-induced decreases in t-PA synthesis were counteracted by (pre-)incubation of the endothelial cells with 100 μg/mL genistein (Table 1). Cell viability was not affected by the various concentrations of genistein used.

Genistein inhibits mitogenesis by inhibiting tyrosine phosphorylation. We evaluated whether inhibition of mitogenesis and inhibition of PAI-1 synthesis by genistein were coupled. Endothelial cell growth was stimulated by b-FGF. Figure 2A shows that various tyrosine protein kinase inhibitors (genistein, tyrphostin A47,[43] herbimycin A,[44] the stable erbstatin analogue methyl-2,5-dihydroxy-cinnamate,[45] and the thiazolidine-dione compound 5[46]) reduced b-FGF–induced [^{3}H]thymidine incorporation in human endothelial cells. However, whereas herbimycin A, tyrphostin A47, methyl-2,5-dihydroxy-cinnamate, and compound 5 were as least as active as genistein in inhibiting [^{3}H]thymidine incorporation, only genistein and herbimycin A reduced PAI-1 synthesis in TNFα-treated endothelial cells (Fig 2B).

To obtain further evidence for the involvement of tyrosine phosphorylation in the regulation of PAI-1 synthesis, endothelial cells were incubated with various concentrations of vanadate. Figure 2C shows that PAI-1 synthesis increased in a concentration-dependent way after incubation with vanadate. Simultaneous measurement of the incorporation of [^{35}S]methionine in trichloroacetic acid-soluble products showed that this increase was not caused by a change in overall protein synthesis. Vanadate did not affect the overall protein synthesis at concentrations up to 30 μmol/L (TNFα-treated cells) or 100 μmol/L (control cells) (not shown).

To investigate if genistein affected the production of PAI-1 mRNA, RNA was extracted from cells 6 hours or 18 hours after addition of TNFα and/or genistein, and PAI-1 mRNA quantities were evaluated from Northern blots by autoradiography and density scanning (Fig 3). TNFα induced a marked increase in the two PAI-1 mRNAs, the 3.0-kb species being slightly more elevated than the 2.3-kb form. A profound decrease in PAI-1 mRNA was observed when the cells were simultaneously incubated with 30 or 100 μg/mL genistein. Genistein also decreased the PAI-1 mRNA under basal con-

Table 1. Effect of Genistein on the Production of PAI-1, u-PA, and t-PA by Human Endothelial Cells

Addition	PAI-1 Antigen % of Control	u-PA Antigen % of Control	t-PA Antigen % of Control
None	≡100 (138 ± 21 ng/10⁵ cells)	≡100 (0.25 ± 0.16 ng/10⁵ cells)	≡100 (2.5 ± 0.9 ng/10⁵ cells)
Genistein (100 μg/mL)	50 ± 2*	103 ± 26	88 ± 7
TNFα (500 U/mL)	418 ± 93†	952 ± 131	62 ± 4
TNFα + genistein	98 ± 8*	917 ± 144	96 ± 3
LPS (10 μg/mL)	337 ± 54†	416 ± 45	69 ± 3
LPS + genistein	88 ± 10*	385 ± 57	102 ± 22
IL-4 (200 U/mL)	145 ± 18‡	95 ± 15	93 ± 12
IL-4 + genistein	56 ± 4*	118 ± 27	81 ± 9

The production of PAI-1, t-PA, and u-PA antigens was assayed in the endothelial cell-conditioned medium after a 24-hour incubation period in M199-10% human serum. Data represent the mean ± SEM of four independent cultures (confluent, after two passages). Genistein (100 μg/mL) was added 1 hour before the incubation period and remained present during incubation. The effect of IL-4 was inhibited by specific IL-4 antibodies and not influenced by addition of polymyxin B.

Statistical significance was determined by the *t*-test for paired data: *$P < .05$ compared to without addition of genistein; ‡$P < .01$; †$P < .05$ compared to no addition.

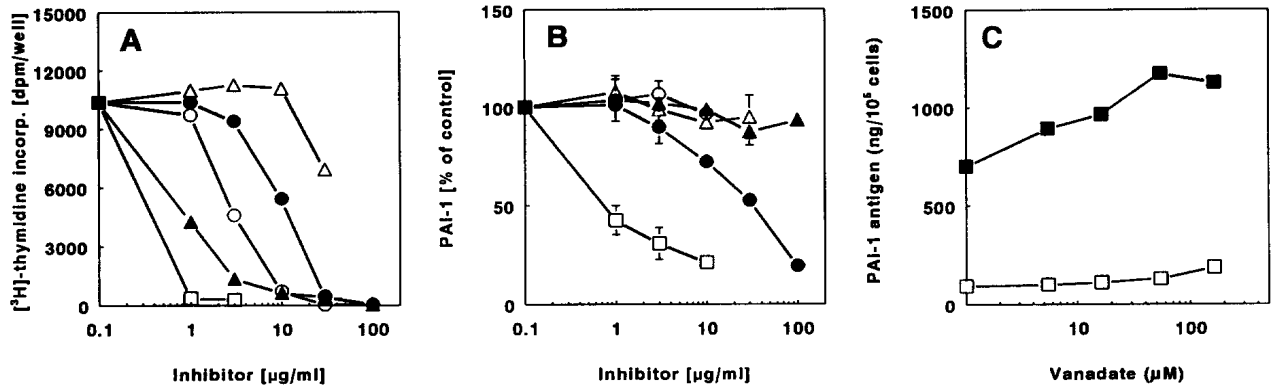

Fig 2. Effect of various tyrosine protein kinase inhibitors on b-FGF–induced [³H]thymidine incorporation and TNFα-induced PAI-1 synthesis by human endothelial cells. Genistein (●), tyrphostin A47 (▲), herbimycin A (□), or compound 5 (△) did not affect cell viability or morphology at all concentrations tested; the erbstatin-analogue methyl-2,5-dihydroxy-cinnamate (○) was toxic for the cells above 10 to 30 μg/mL. (A) Mitogenesis was stimulated in subconfluent cells by addition of 20 ng/mL b-FGF and various concentrations of protein tyrosine kinase inhibitors (●, ▲, △, ■, ○). After 18 hours, a tracer amount of [³H]thymidine was added to the medium and incubation continued in the same medium for another 6 hours. Subsequently [³H]thymidine incorporation was determined as described in Materials and Methods. (B) The effect of the same protein tyrosine kinase inhibitors on the induction of PAI-1 antigen by 500 U/mL TNFα was evaluated after a 24-hour period as described in the legend of Table 1. Data are mean ± SEM of three independent experiments; control values were 615 ± 145 ng/10⁵ cells. (C) The effect of the tyrosine protein phosphatase inhibitor vanadate on PAI-1 production was measured in conditioned medium of cells that had been preincubated for 20 minutes with vandate and subsequently incubated for 24 hours with the same concentration of vanadate together with 500 U/mL TNFα (■) or without it (□).

ditions; it had no effect on the GAPDH mRNA under basal or TNFα-stimulated conditions.

Transcriptional activation of the PAI-1 gene by TNFα was shown by run-on assay (Fig 4). Four hours after addition of TNFα the transcription rate of PAI-1 was elevated sevenfold to ninefold, as determined by density scanning of the slot blots. Transcription of u-PA was hardly detectable under basal conditions; the amount of u-PA transcripts in run-on assay tended to be higher in the TNFα-treated cells. Transcription rates of GAPDH, 55-kD TNF-receptor (TNFR-p55), 75-kD TNF-receptor (TNFR-p75), and actin remained unaltered in nuclei of TNFα-treated cells and basal cells. Genistein prevented the increase in PAI-1 transcription, indicating that it acted on an event related to or preceding gene transcription of PAI-1. It also decreased the basal transcription of PAI-1, albeit to a lesser extent than that of the TNFα-induced increase of PAI-1. Genistein did not markedly change the transcription rates of u-PA, t-PA, u-PA receptor, GAPDH, or actin in TNFα-treated endothelial cells; it increased the transcription rates of both TNF-receptors by 30% to 70%.

TNFα interacts with cells via two different receptors, a 55-kD TNF-receptor and a 75-kD TNF-receptor.[47] To test the possibility that the different effects of genistein on PAI-1 and u-PA synthesis were caused by the involvement of different TNF-receptors, we used a TNFα mutant $Trp^{32}Thr^{86}$TNFα, which selectively activates the 55-kD TNF-receptor.[27] Both PAI-1 and u-PA synthesis were increased after selective activation of the 55-kD TNF-receptor (Fig 5). Simultaneous incubation with genistein reduced the increase in PAI-1 synthesis but not the u-PA induction.

DISCUSSION

Inflammatory mediators can markedly enhance PAI-1 production in vitro[13-19] and in vivo.[13,14,16,19-22] In the present study we found that genistein can inhibit TNFα-induced transcription and synthesis of PAI-1 by human endothelial cells in vitro, but not that of u-PA. Genistein was rather selective in this effect, because several other tyrosine kinase inhibitors were unable to inhibit PAI-1 synthesis.

Genistein acted on the basal PAI-1 synthesis and, more potently, on TNFα-stimulated PAI-1 synthesis. Apparently it did not cause a general reduction of endothelial cell activation by TNFα, because it did not affect the TNFα-induced production of u-PA. The different responses of PAI-1 and u-PA to genistein may be due to the involvement of different TNF receptors in the induction of PAI-1 and u-PA or to different regulation of PAI-1 and u-PA transcription and protein synthesis. A differential effect of genistein on the two TNF-receptors did not cause the different effects of genistein on PAI-1 and u-PA synthesis, because induction of both PAI-1 and u-PA was achieved by a selective 55-kD TNF-receptor agonist, and similar effects of genistein were then found. On the other hand, genistein markedly reduced the transcription rate of PAI-1, whereas it did not affect the transcription rate of u-PA. Therefore, genistein acts selectively on a pathway that stimulates the activation of the PAI-1 gene transcription.

Additional evidence was obtained that the signal transduction pathways for TNFα-induced synthesis of PAI-1 and that of u-PA diverge after receptor activation. Ro31-8220, a specific inhibitor of protein kinase C,[48] had a profound effect on u-PA synthesis and only a moderate effect on PAI-1 synthesis by human endothelial cells (N.C.A.J. van de Kar, T. Kooistra, and V.W.M. van Hinsbergh, unpublished data, August 1993). These observations are in agreement with previously reported data showing the involvement of protein kinase C in TNFα-induced u-PA synthesis[49] and the relative insensitivity of endothelial PAI-1 synthesis to downregulation of protein kinase C.[50] Thus, a direct inhibition of protein

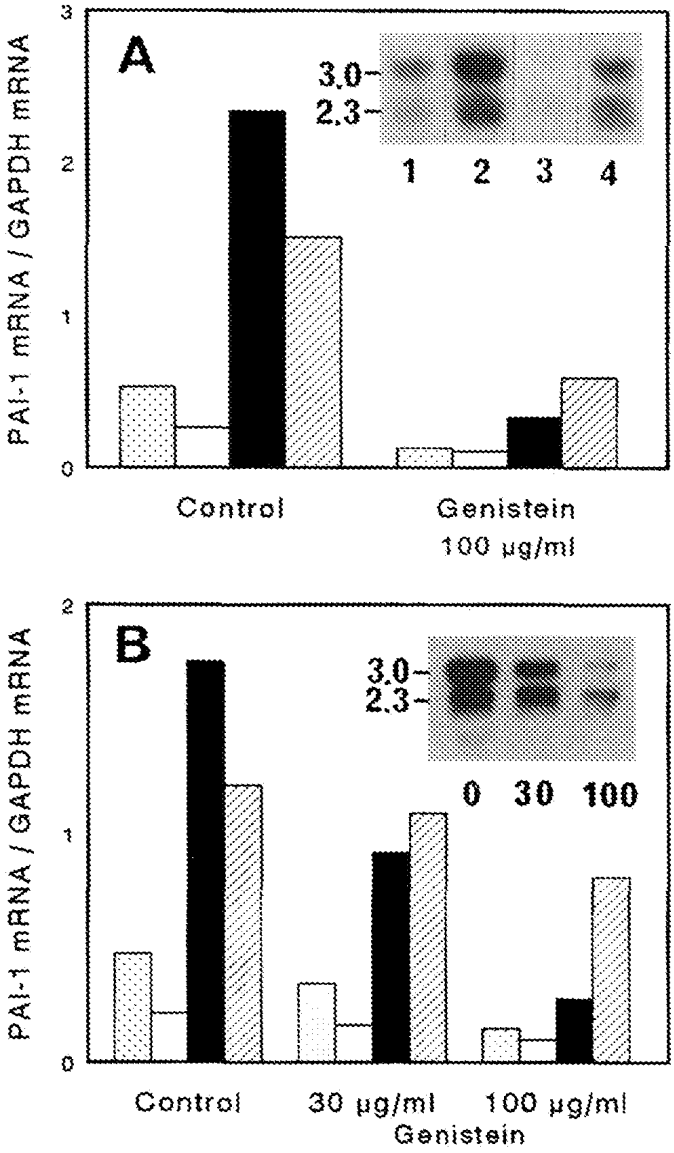

Fig 3. Effect of genistein on PAI-1 mRNA. Human umbilical vein endothelial cells were incubated for 6 hours (A) or 18 hours (B) with the indicated amount of genistein in M199-10% human serum supplemented with 500 U/mL TNFα or without it. The inset of (A) shows the effect of TNFα and genistein on the two mRNAs (2.3 and 3.0 kb) of PAI-1 in human umbilical vein endothelial cells: 1, control; 2, TNFα; 3, genistein; 4, TNFα + genistein. The amount of PAI-1 mRNA was estimated by scanning of the density of the PAI-1 mRNA bands and GAPDH mRNA band (as internal standard) on the autoradiograms, and is represented in the bar graph. (▨) 3.0-kb PAI-1 mRNA; control; (□) 2.3-kb PAI-1 mRNA, control; (■) 3.0-kb PAI-1 mRNA, TNFα-treated; (▩) 2.3-kb PAI-1 mRNA, TNFα-treated. (B) Concentration dependency of the effect of genistein on PAI-1 mRNA is given for TNFα-treated cells in the inset; 0, 30, and 100 refer to the genistein concentration in micrograms per milliliter. Densitometric scanning of the mRNAs is given in the bar graph similarly as described under (A). (A) and (B) were obtained with different cultures.

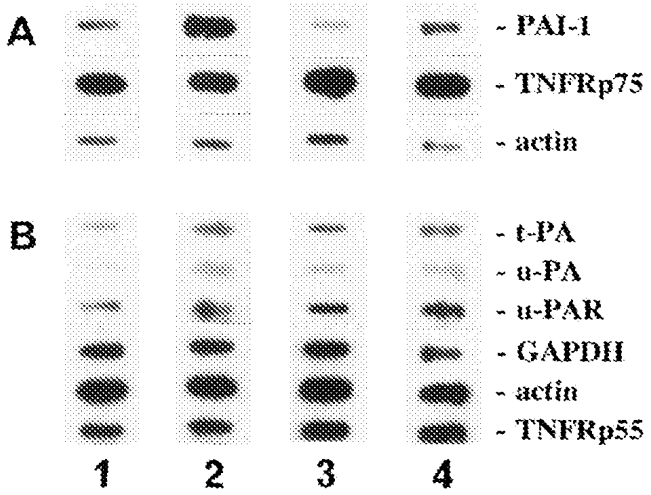

Fig 4. Transcription rates of various endothelial cell proteins was determined by run-on assay in nuclei of human umbilical vein endothelial cells 4 hours after addition of 500 U/mL TNFα (lanes 2 and 4) and in their untreated counterparts (lanes 1 and 3). Genistein (100 µg/mL) was added 1 hour before the incubation and remained present during the 4-hour incubation (lanes 3 and 4). The figures represent autoradiograms of the slot blots containing the indicated species of cDNA to which the radiolabeled RNA was hybridized. The autoradiographs of (A) were obtained after an 18-hour exposure of the films; those of (B) were exposed for 7 days. Experimental details are described in Materials and Methods. TNFR-p55 and TNFR-p75 indicate the two TNF receptors, u-PAR indicates the u-PA receptor.

kinase C, which phosphorylates at serine/threonine instead of tyrosine, is unlikely to be involved in genistein inhibition of PAI-1 transcription.

Genistein has been widely used as a tyrosine protein kinase inhibitor[51] and inhibited b-FGF–induced mitogenesis of endothelial cells that involves tyrosine protein kinase activity[52,53] (and this study). Fotsis et al[25] have reported that genistein inhibits basal– and b-FGF–induced production of PAI-1 in bovine endothelial cells. They suggested that one of the possible mechanisms of genistein action on these parameters may be inhibition of the tyrosine protein kinase activity of the b-FGF receptor itself. A similar mechanism, inhibition of tyrosine protein kinase activity of the insulin receptor, has been proposed by Schneider et al[54] for the repression of PAI-1 synthesis by genistein in insulin-stimulated cells. In our experiments, receptor-coupled tyrosine protein kinase activity seems not likely to underlie the inhibition of PAI-1 synthesis by genistein. Several mediators, TNFα, LPS, IL-1α, IL-4, and thrombin, induce PAI-1 production in human endothelial cells. In all cases the induced PAI-1 production is inhibited more strongly than the basal PAI-1 production by genistein. Furthermore, inhibition of PAI-1 synthesis occurs selectively by genistein, whereas several other tyrosine kinase inhibitors, including tyrphostin A47 and compound 5, which usually inhibit receptor-linked tyrosine protein kinase activity, appear not to inhibit PAI-1 synthesis. On the other hand, these tyrosine protein kinase inhibitors did inhibit b-FGF–induced mitogenesis in human endothelial cells. This indicates that the tyrosine protein ki-

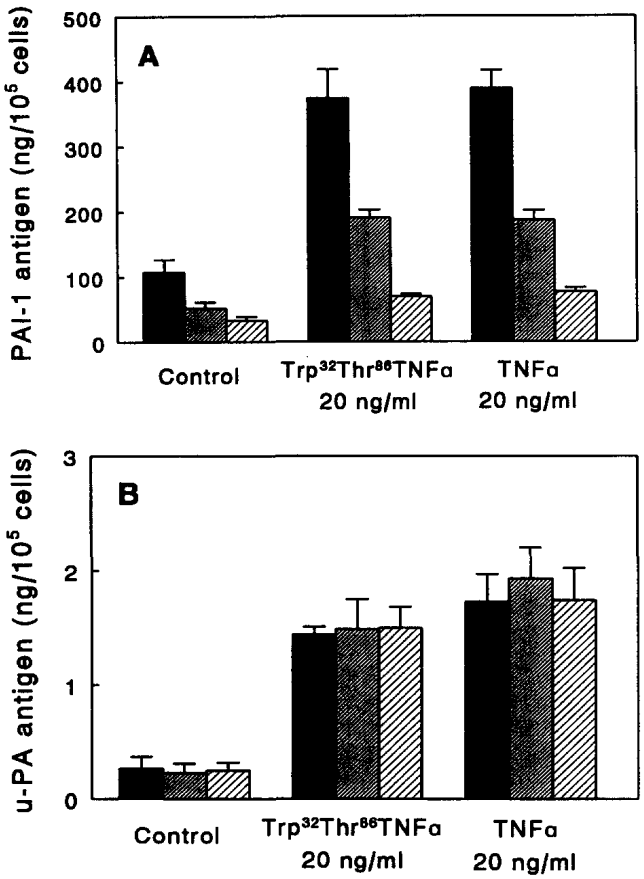

Fig 5. The induction of PAI-1 by the $Trp^{32}Thr^{86}TNF\alpha$ mutant, which only recognizes the 55-kD TNF-receptor (TNFR-p55), is inhibited by genistein, whereas the production of u-PA remains unaltered. The production of PAI-1 and u-PA antigens were assayed after 24-hour incubation of human umbilical vein endothelial cells in M199 medium-10% human serum, supplemented with 20 ng/mL TNFα, 20 ng $Trp^{32}Thr^{86}TNF\alpha$ mutant, or with no further addition (control). Genistein (30 μg/mL [▨]; 100 μg/mL [▨]; no addition [■]) was added 1 hour before the incubation period and remained present during incubation. The data represent the mean ± SEM of three different cultures.

nase activity, which is necessary for the induction of mitogenesis, is not involved in PAI-1 induction.

Two alternative explanations may account for the effect of genistein on PAI-1 synthesis. Genistein may act on PAI-1 synthesis by an effect unrelated to its action on protein phosphorylation or it may act on a specific protein phosphorylation, which is rather insensitive to most of the other tyrosine protein kinase inhibitors tested. The first possibility is unlikely, because the structural analogue daidzein, which differs only from genistein by the lack of a hydroxylic group at the carbon-5 position (a change that results in a profound loss of tyrosine protein kinase inhibiting capacity[42]) did not reduce PAI-1 synthesis. The findings that herbimycin A, another generally used tyrosine protein kinase inhibitor, also reduce PAI-1 synthesis, and that inhibition of tyrosine protein phosphatase(s) by vanadate caused the opposite response, give additional evidence for the involvement of tyrosine protein phosphorylation in the regulation of PAI-1 synthesis.

Several nonreceptor proteins have been mentioned as being affected by genistein, including topoisomerases I and II,[54] which can affect transcription,[54,55] and S6 kinase in mitogen-stimulated cells.[56] Furthermore, genistein decreased c-*myc* mRNA in NIH-3T3 cells at a similar concentration as it inhibits PAI-1 synthesis in endothelial cells.[55] c-*Myc* has been implicated in the posttranscriptional regulation of PAI-1.[57,58] However, the effect of genistein on endothelial PAI-1 synthesis occurred during or before transcriptional activation of the PAI-1 gene.

These observations lead us to conclude that a genistein-sensitive phosphorylation step is involved in the signal transduction pathway, which occurs between TNF-receptor activation and transcription of PAI-1. The unique properties of the genistein-sensitive pathway of PAI-1 induction make it a potential target for pharmacologic intervention.

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